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# MiR-874 promotes intestinal barrier dysfunction through targeting AQP3 following intestinal ischemic injury

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ABSTRACT

**Intestinal ischemic injury is a significant clinical problem arising from diseases or as a complication of abdominal surgery. Our previous study showed aquaporin 3 is involved in intestinal barrier impairment. Here, we revealed that intestinal ischemia induced a time-dependent increase of miR-874 expression and a time-dependent decrease of AQP3 expression, and the level of miR-874 expression was inversely related to AQP3 protein expression. In addition, miR-874 promoted the paracellular permeability *in vitro* through targeting 3′UTR of *AQP3*. Two of the tight junction proteins, Occludin and Claudin-1, were found to be involved in miR-874-induced intestinal barrier dysfunction.**



## 1. Introduction

Ischemic injury is observed in a variety of diseases, such as vascular occlusion, haemorrhagic shock or trauma [1,2]. Many lines of evidence support the concept that ischemic injury may initiate a series of intestinal reactions such as intestinal barrier dysfunction, translocation of intestinal bacteria, or even cause systemic inflammatory response syndrome (SIRS) [3]. The mechanism of intestinal mucosal barrier is very complicated, which may constitute of mechanical barrier, immune barrier, chemical barrier and biological barrier [4]. However, the underlying molecular mechanism of the intestinal barrier dysfunction following ischemia has remained poorly understood.

AQPs (aquaporins) are a family of small (30 kDa/monomer) hydrophobic, integral membrane proteins, which belong to a special superfamily of MIPs (major intrinsic proteins) [5]. AQP3 is mainly expressed in the epithelia of the upper and lower digestive tract and acts as membrane channels for the selective transport of water, glycerol and other small solutes across the cell membrane [6]. We recently demonstrated that down-regulation of AQP3 in Caco-2 cells contributed significantly to impair the intestinal barrier integrity via opening the tight junction complex. [7]. However, the mechanism by which AQP3 is regulated and the expression level of AQP3 after ischemic injury are still unclear.

MicroRNAs are approximate 20 nucleotides RNA molecules that play a role in modulating the activity of thousands of genes [8]. Recently, a role for microRNAs in the regulation of intestinal barrier has begun to be explored. McKenna et al. [9] demonstrated that employing gene ablation of *Dicer 1* induced intestinal barrier dysfunction and inflammation in mice. Our previous study showed that miR-874 suppressed AQP3 expression by binding to the 3′UTR of *AQP3* mRNA in gastric cancer cells [10]. In this study, we investigated the expression pattern of miR-874 and AQP3 in mice with intestinal ischemic injury and then confirmed the effect of miR-874 on the regulation of intestinal barrier through targeting AQP3.

## 2. Materials and methods

### 2.1. Intestinal ischemic injury

C57BL/6 mice were purchased from the Shanghai Experimental Animal Center (Chinese Academy of Sciences, Shanghai, China) and housed under controlled conditions of temperature and humidity. Forty 8-week-old mice were randomized into sham group, ischemic group (15 min), ischemic group (30 min) and ischemic group (45 min). After intraperitoneal anesthesia with 40 mg/kg

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pentobarbital, the abdomen was opened and the superior mesenteric artery (SMA) was occluded for 15, 30 or 45 min using non-traumatic vascular clamps in each ischemic group without reperfusion. Animals in the sham group underwent identical procedures without SMA occlusion. After those procedures, 10 cm of jejunum was obtained for western blot and quantitative real-time PCR analyses. All experiments were approved and monitored by the institutional animal care and use committee of Nanjing Medical University.

### 2.2. Cell culture assays

Caco-2 cells and LS174T cells were purchased from Chinese Academy of Sciences and were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco, Uruguay), 100 mg/ml penicillin and 100 mg/ml streptomycin (Gibco, NY, USA) at 37 °C with 5% $CO_2$. To form the Caco-2 monolayer, $2 \times 10^5$ Caco-2 cells were seeded onto the apical chamber of Transwell bicameral chamber containing 0.4 μm pores. The cells were grown for 14 days to allow them to reach confluence and fully differentiate. The medium was changed every other day.

Hypoxia was induced *in vitro* by incubating the cells in anaerobic chambers. Cells were placed in an airtight container (Thermo Scientific) and maintained at 37 °C in humidified atmosphere containing 1% $O_2$ and 10% $CO_2$. Media were equilibrated for 24 h to the required oxygen level in the hypoxia workstation in the same conditions before use.

### 2.3. Lentivirus production and cell transfection

We modified the commercial LV3-has-miR-874-premicroRNA vector (pre-miR-874) and LV3-has-miR-874-sponge inhibitor vector (miR-874 inhibitor) lentiviral constructs (Genepharma, ShangHai, China) to overexpress or knockdown miR-874 in Caco-2 and LS174T cells. LV3 empty lentiviral construct (miR-NC) served as a negative control. Retrovirus packaging and infection were conducted as described previously [10].

### 2.4. Luciferase assay

The 3′UTR of *AQP3* complementary DNA containing the wild-type or mutated miR-874 binding sequences were synthesized by Shenggong (Shanghai, China). The sequence was cloned into the FseI and XbaI restriction sites of the pGL3 luciferase control reporter vector (Promega, USA) to generate the *AQP3* 3′UTR reporter. Total $5 \times 10^5$ Caco-2 and LS174T cells stably transfected with pre-miR-874 or miR-NC were seeded in 24-well plates. Cells were transfected with 0.12 μg of either pGL3-WT-AQP3 or pGL3-MUT-AQP3 3′UTR reporter plasmid. Meanwhile, 0.01 μg of Renilla luciferase expression plasmid was also transfected into the above cells as a reference control. Firefly and Renilla luciferase activities were measured by Dual-Luciferase reporter assay (Promega, E1910, WI, USA) at 36 h after transfection according to the manufacturer's instruction. The relative luciferase activity was calculated as firefly fluorescence/Renilla fluorescence.

### 2.5. Quantitative real-time PCR

Quantitative real-time PCR was performed as previously described [10]. TaqMan gene expression assays for murine and human AQP3 and specific TaqMan probes (Genepharma, ShangHai, China) to quantify miR-874 were used. Gene expression levels were normalized to the level of β-actin and snRNA U6. Results were calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were done independently in triplicate.

### 2.6. Western blot assay

Antibodies against AQP3 (Abcam), Clauding-1 (Cell Signalling Technology), Occludin (Abcam), Claudin-2 (Abcam), Zo-1 (Cell Signalling Technology), E-cadherin (Cell Signalling Technology) and beta-catenin (Cell Signalling Technology) were used. Protein were separated on the 10% SDS polyacrylamide gel and transferred to 0.2-mm nitrocellulose membrane which was then blocked in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% milk. Blots were incubated with specific first antibodies in dilution buffer at 4 °C overnight. The blotted membranes were then incubated with HRP-conjugated anti-mouse or antirabbit IgG (Cell Signalling Technology) at room temperature for 2 h. Targeting protein expression levels were detected using an enhanced chemiluminescence (ECL) detection system following the manufacturer's instructions. GAPDH was used as an internal control.

### 2.7. Measurement of the transepithelial electrical resistance

The formation of a monolayer of Caco-2 cells was monitored by the measurements of the transepithelial electrical resistance (TEER) with a Millicell electrical resistance system (Millipore, Bedford, MA) as described previously [7]. Briefly, immerse the electrodes so that the shorter electrode is in the Millicell culture plate insert and the longer electrode is in the outer well. The shorter electrode should not contact cells growing on the membrane. All experiments were done independently in triplicate.

### 2.8. Detection of paracellular permeability

Monolayers were used in this experiment only after the TEER had risen above 150 $\Omega/cm^2$. Generally, about 14 days of culture are needed to reach such a TEER. Paracellular permeability was assessed by measuring the flux of lucifer yellow (LY) from apical to basal chambers of the Transwell [11]. LY was diluted in transport buffer (HBSS with $Ca^{2+}$, $Mg^{2+}$, +10 mM HEPES, pH 7.4) and added to the apical chamber at a final concentration of 50 μM. Three hours later, LY concentration in the basal chamber was measured with TECAN Infinite F500 (Männedorf, Switzerland) luminescence spectrometer against a standard concentration curve (excitation 427 nm, emission 536 nm).

### 2.9. Bacterial translocation

The DMEM used to culture the cells was removed from the bicameral chamber and replaced with fresh HBSS. An inoculums containing $10^5$ bacteria (*Escherichia coli* C25) was added to the apical side of the Caco-2 cell monolayer that had been grown in culture for 14 days. Bacterial translocation was evaluated by quantitatively culturing samples of medium (100 μl) obtained from the basal chamber at 20, 40, 120, and 180 min after adding bacteria. The number of viable translocation *E. coli* C25 was quantitated by pour late assay with Macconkey's agar.

### 2.10. Statistical analysis

Data are expressed as means ± S.D. Differences were analysed with the unpaired Student's *t* test and Analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant. Statistics were performed using SPSS version 19.0 for windows.

## 3. Results

### 3.1. Changes of AQP3 and miR-874 expression in a mouse model of acute intestinal ischemia

Western blot and quantitative real-time PCR were performed to detect the AQP3 and miR-874 expression. Results showed that ischemia induced a time-dependent increase of miR-874 expression and a time-dependent decrease of AQP3 expression (Fig. 1A). Notably, we found that the level of miR-874 expression was inversely related to AQP3 protein expression after 30 min of intestinal ischemia (Fig. 1B). These data indicated that miR-874 might have an impact on AQP3 expression and intestinal barrier.

### 3.2. Expression pattern of miR-874 and AQP3 in Caco-2 cells under hypoxic or serum-free conditions

Similarly, the protein level of AQP3 was significantly reduced and the level of miR-874 was increased in a time-dependent manner in condition of hypoxia or serum-free (Fig. 1C and D). In addition, the variation of miR-874 and AQP3 seemed more sensitive to hypoxia. After 8 h of hypoxia, the level of miR-874 and AQP3 showed a tendency towards stabilization (Fig. 1C and D). Correlation analysis further suggested that the level of miR-874 expression was inversely related to AQP3 protein expression in condition of hypoxia. The expression of miR-874 and AQP3 showed a tendency towards correlation, but did not get a significant *P* value (Fig. 1E).

### 3.3. miR-874 directly interacts with the 3′UTR of AQP3 in Caco-2 and LS174T cells

Our previously study revealed that miR-874 decreased AQP3 expression in gastric cancer cells by targeting its 3′UTR [10]. Bioinformatic analysis showed the miR-874 binding site in 3′UTR of *AQP3* (Fig. 2G). Wild-type (WT) and mutant (MUT) 3′UTR of *AQP3* were cloned into the luciferase reporter. The fluorescent activity of the reporter was significantly declined in the cells that were transfected with pGL3-WT-AQP3 3′UTR in Caco-2 cells and LS174T cells stably transfected with pre-miR-874 (Fig. 2H and Fig. S1). The fluorescent activity did not change significantly in the cells transfected with pGL3-MUT-AQP3 3′UTR.

To further investigate the impact of miR-874 on AQP3 expression in Caco-2 and LS174T cells, we performed both miR-874 overexpression and knockdown experiments. Silence of miR-874 resulted in the up-regulation of AQP3 protein compared with the negative control. Conversely, the protein level of AQP3 was significantly down-regulated after transfection with pre-miR-874 (Fig. 2H).

### 3.4. miR-874 inhibits MUC2 expression in LS174T cells

MUC2 has been proven to play an important role in intestinal barrier. We investigated that MUC2 was significantly inhibited after miR-874 overexpression in LS174T cells (Fig. 2A), which was a ideal *in vitro* model for studying MUC2 expression [12]. The results indicated miR-874 might also affect mucus barrier.

### 3.5. miR-874 promotes intestinal barrier dysfunction in vitro

Overexpression of miR-874 in Caco-2 cells caused an increase of paracellular permeability, as measured by decreased TEER and increased LY permeability (Fig. 2B and C). These effects were time-dependent.

*E. coli* translocation assay was performed after the cells had grown for 14 days to allow them to reach confluence and fully differentiate. We found that Caco-2 cells with miR-874 overexpression showed more *E. coli* translocation than negative control at each time point (Fig. 2D).

TNF-α is a main factor to mediate the intestinal barrier dysfunction. We observed the role of miR-874 in TNF-α-mediated intestinal barrier dysfunction. Overexpression of miR-874 in Caco-2 cells promoted the increase of paracellular permeability caused by TNF-α (Fig. 2E and F).

### 3.6. miR-874 alters the expression of the tight junction proteins

The tight junction proteins were detected by western blot to investigate the mechanism of miR-874-induced intestinal barrier dysfunction with or without TNF-α. The results showed that Occludin and Claudin-1 were significantly increased in Caco-2 cells treated with miR-874 inhibitor compared with the negative control. By contrast, miR-874 overexpression suppressed Occludin and Claudin-1 expression. Expression of the other tight junction proteins, such as ZO-1, Claudin-2, E-cadherin and beta-catenin, did not change significantly (Fig. 3). TNF-α had little effect on the expression of tight junction proteins.

## 4. Discussion

Intestinal ischemic injury is a significant clinical problem arising from diseases or as a complication of abdominal surgery [13]. The intestinal mucosa is particularly susceptible to ischemic injury. Intestinal barrier dysfunction resulting from mucosal ischemia has also been observed and might contribute to the progression of intestinal lesions, or even cause systemic inflammatory response syndrome (SIRS) [14]. A firm association between AQP3 and intestinal barrier dysfunction has been established in our previous study [7]. Recently, a role for microRNAs in the regulation of intestinal barrier has begun to be explored [9,15]. Silence of *Dicer 1* caused intestinal barrier dysfunction and inflammation in mice [9]. Our previous study showed that miR-874 suppressed AQP3 expression by binding to the 3′UTR of *AQP3* mRNA in gastric cancer cells [10]. In this study, we investigated the expression pattern of miR-874 and AQP3 in a mouse model of acute intestinal ischemia, and then confirmed the effect and underlying mechanism of miR-874 on regulation of intestinal barrier. Our results revealed that ischemia induced a time-dependent increase of miR-874 expression and a time-dependent decrease of AQP3 expression, and the level of miR-874 expression was inversely related to AQP3 protein expression. In addition, miR-874 promoted the paracellular permeability *in vitro* through targeting 3′UTR of *AQP3*. Two of the tight junction proteins, Occludin and Claudin-1, were found to be involved in miR-874-induced intestinal barrier dysfunction.

Most intestinal mucosal surfaces are covered by a hydrated gel formed by MUC2, which creates a barrier that prevents large particles, including most bacteria, from directly contacting the epithelial cell layer [16]. Grootjans et al. [17] were the first to show that colonic ischemia lead to loss of the mucus layer and consequently allowed adherence of bacteria to the epithelium and translocation of potential pathogens and their toxins to the internal milieu. In the present study, the expression of MUC2 in LS174T cells was significantly suppressed by miR-874 overexpression. We searched TargetScan, PicTar and miRanda databases to confirm whether MUC2 contains putative targets of miR-874. We did not find any correlation between MUC2 and miR-874. However, we found that miR-874 could bind to the 3′UTR of STAT3, which was crucial to production of MUC2 in LS174T cells by suppressing protein misfolding and endoplasmic reticulum stress. So we assumed that intestinal ischemia and hypoxia induced miR-874 up-regulation, resulting in STAT3 inhibition through targeting its 3′UTR,

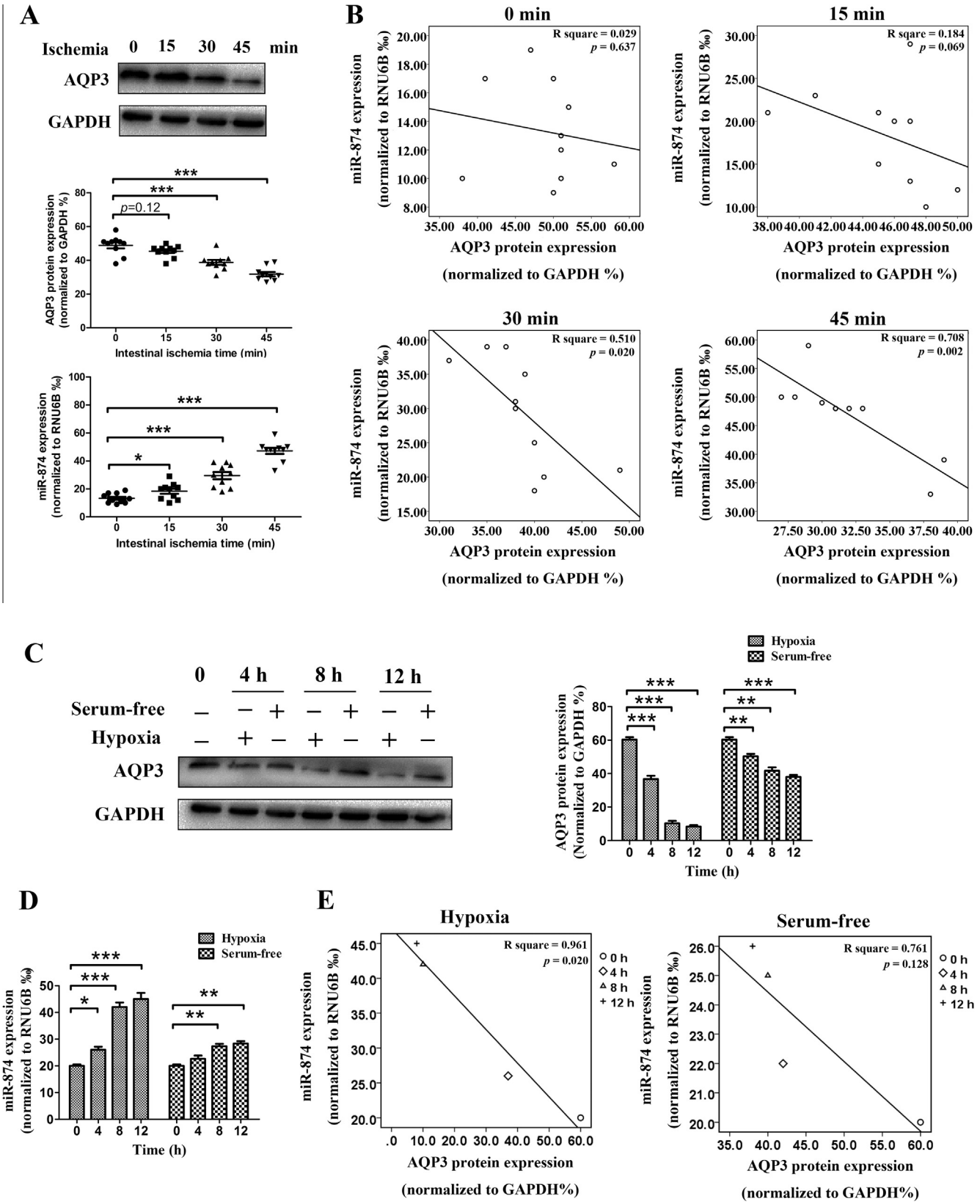


**Fig. 1.** Expression pattern of miR-874 and AQP3 in a mouse ischemia model or cellular hypoxia model. (A) The superior mesenteric artery (SMA) was occluded for 15, 30 and 45 min using non-traumatic vascular clamps in the mouse ischemia model. Each group included 10 mice. Western blot and quantitative real-time PCR were used to detect the expression of miR874 and AQP3. (B) The scatter plots show the expression of miR-874 and AQP3 in each group. Linear regression analysis was used to measure the association between miR-874 and AQP3. (C–E) Hypoxia was induced *in vitro* by incubating the Caco-2 cells in anaerobic chambers containing 1% $O_2$ and 10% $CO_2$. Western blot and quantitative real-time PCR were used to detect the expression of miR874 and AQP3. Linear regression analysis was used to measure the association between miR-874 and AQP3. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

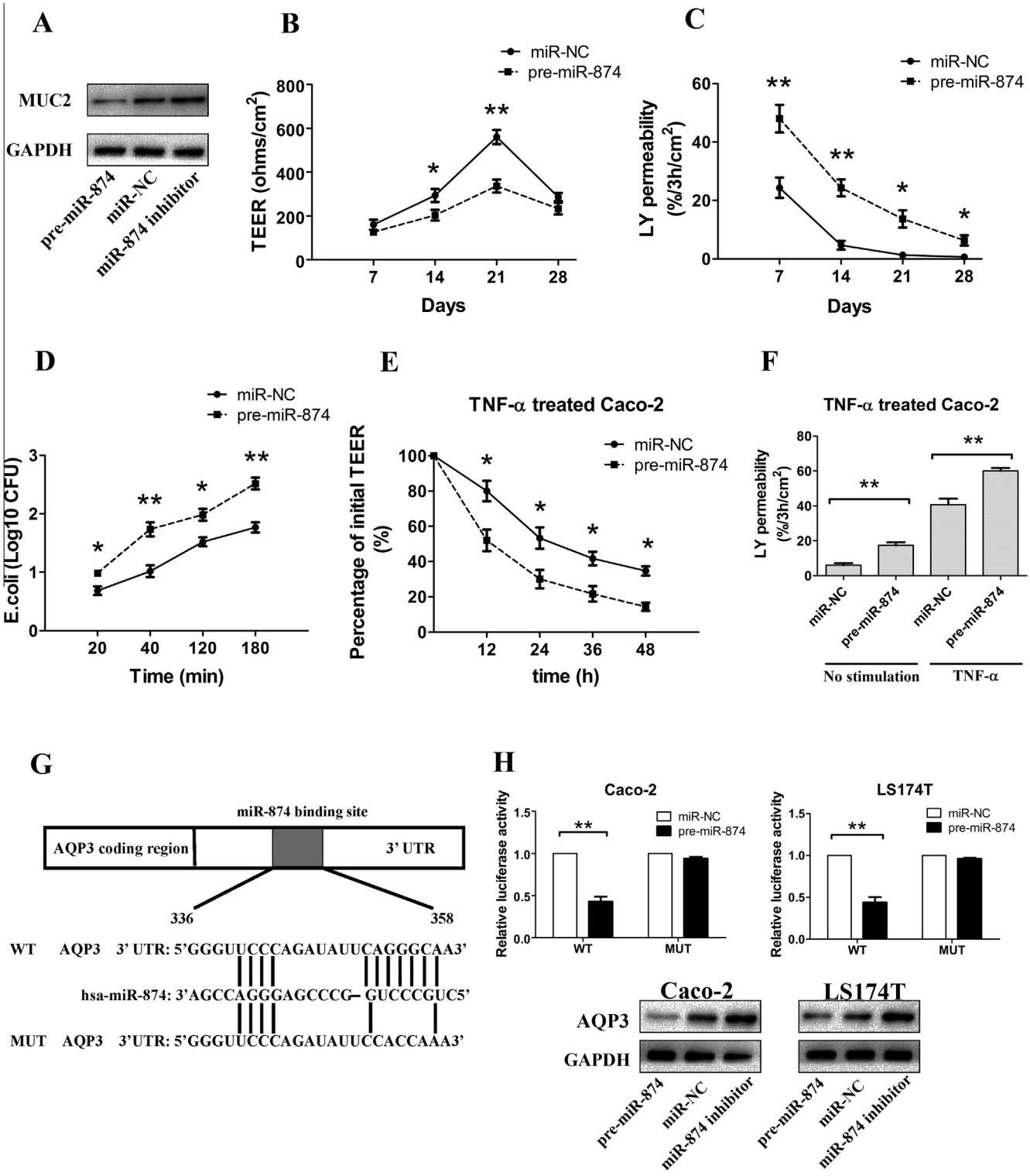


**Fig. 2.** Intestinal barrier dysfunction caused by miR-874. (A) MUC2 was inhibited by overexpression of miR-874. (B–D) The formation of a monolayer of Caco-2 cells was monitored by the measurements of the transepithelial electrical resistance (TEER), lucifer yellow (LY) permeability and bacterial translocation. (E and F) TNF-α was added after the cells had grown for 14 days. Overexpression of miR-874 in Caco-2 cells promoted the increase of paracellular permeability caused by TNF-α. (G) The AQP3 3′UTR regions containing the wild-type or mutant binding site for miR-874 are shown. (H) Relative AQP3 luciferase activity was analyzed after the wild-type or mutant 3′UTR reporter plasmids were transfected into Caco-2 and LS174T cells stably transfected with miR-NC or pre-miR-874. Western blot was used to analyze the expression levels of AQP3. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

and consequently caused the low yields of MUC2 in LS174T cells. However, the underlying mechanism remains further studies.

Tumor necrosis factor-α (TNF-α) is an essential mediator of inflammation in the gut [18,19]. In addition, TNF-α has been shown to cause an increase in intestinal tight junction permeability, which allows increased intestinal penetration of luminal antigens [20–22]. However, the interaction between intestinal ischemia and TNF-α remains unclear. We have developed a

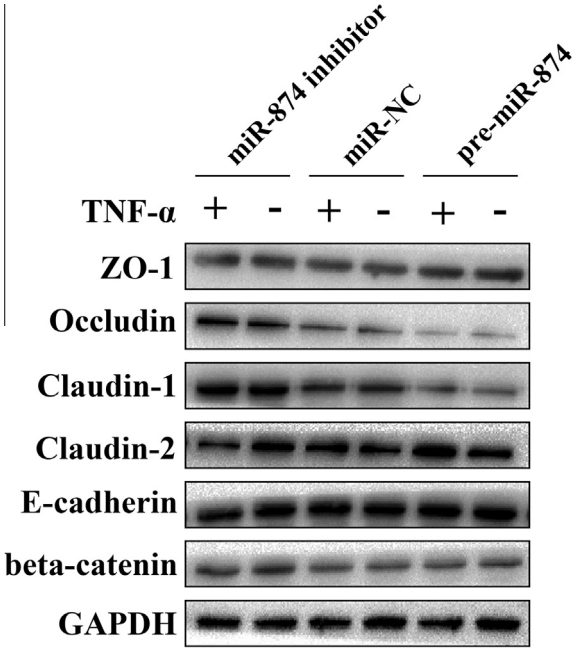


**Fig. 3.** The expression of tight junction proteins was detected by western blot. The cells were treated with TNF-α (10 ng/ml) for 12 h.

cultured epithelial monolayer system *in vitro*. Based on TEER and LY permeability results, it appeared that the Caco-2 cells required approximately 14 days of culture to form a tight monolayer. TNF-α was added when the cells formed a tight monolayer. Our results showed that overexpression of miR-874 promoted the increase of paracellular permeability caused by TNF-α, indicating that miR-874 overexpression and inflammation caused by ischemia might interact and promote the damage of intestinal barrier cooperatively. To further investigate the underlying mechanism, we tested the expression of tight junction proteins.

Tight junctions (TJ) are the major determinants of paracellular permeability in the intestinal. Previous studies have revealed that intestinal injury alters the distribution of TJ proteins [23,24]. Enhancement of the TJ barrier prevents the development of intestinal inflammation [25,26]. Occludin is the first TJ protein to be reported [27]. Knockdown of Occludin leads to an increase in intestinal TJ permeability via a non-restrictive manner [28]. In the recent studies, ischemia/reperfusion resulted in the disruption of Claudin-1 and ZO-1 [23,29]. Our results showed Occludin and Claudin-1 were significantly increased in Caco-2 cells treated with miR-874 inhibitor compared with the negative control. By contrast, miR-874 overexpression suppressed Occludin and Claudin-1 expression, which was consistent with previous findings [30,31]. All these findings further confirmed that miR-874 played an important role in intestinal barrier dysfunction in acute intestinal ischemia.

In conclusion, this study demonstrated that intestinal ischemia changed the expression of miR874 and AQP3 in a mouse model. Moreover, miR-874 promoted the paracellular permeability *in vitro* through targeting 3′UTR of *AQP3*. These findings identified miR-874 as a novel therapeutic target. Further studies are required to reveal the possibility of interference of miR-874 expression in the treatment for ischemic disease.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.01.022.

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